3357

The Major Component of Human Casein: A Protein Phosphorylated at Different Levels

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A single protein, with 0–5 atoms of P per molecule, makes up the major portion of human casein. Also present is a fraction similar to bovine kappa-casein in its ability, in the presence of Ca^{2+}, to solubilize the phosphoprotein as micelles.

Human casein, like bovine casein, is made up of several proteins which interact to form the micelles found in milk. The heterogeneity of human casein was first detected in 1945 by Mellander (1) by moving-boundary electrophoresis. Later, Malpress *et al.* (2, 3) reported that casein from mature human milk showed six zones after electrophoresis in starch gel, that fractions resembling bovine α_s- and κ-caseins could be prepared and that rennin acts upon human casein or its κ-fraction to release a glycopeptide comparable to bovine caseino-glycomacropeptide. Alais and Jollès (4) had previously published amino acid and carbohydrate analyses of whole human casein and of the glycopeptide. Even more extensive heterogeneity of human casein was indicated by the experiments of Nagasawa *et al.* (5) who demonstrated 14–26 bands in zone electrophoretic patterns. We wished to compare purified components of human and cow caseins, and, in this paper, report the separation of human casein into its major components, analyses of their composition, and some of their properties. Preliminary reports of some of the data have been made (6).

MATERIALS AND METHODS

Samples of individual mature (2, 3), human milk, which had been frozen and stored for 1–2 years, were thawed, defatted, and then dialyzed overnight against distilled water at 3°. Casein was precipitated at room temperature by the addition of acid to pH 4.6, centrifuged off, washed with water at pH 4.6, and dried by lyophilization. For fractionation it was dissolved in 0.005 M phosphate buffer, pH 8.3, and sufficient 1 N NaOH to restore the pH to 8.3. Conditions for separating and purifying the components by column chromatography are summarized in Table I. After chromatography the proteins were recovered routinely by dialysis against water and lyophilization. The purified caseins, I through VI, were finally redissolved, reprecipitated at the pH of approximate minimum solubility (Table I), centrifuged, washed, and lyophilized. Estimated percentages of the components are also listed in Table I. The κ-fraction was not purified further.

Purity was evaluated and proteins were identified by disc gel electrophoresis (pH 9.6, 4 *M* urea) as described earlier (7).

Tryptophan analyses were made by Procedure U of Spies (8).

Phosphorus was determined by the microphosphate method of Meun and Smith (9).

The procedures described by Moore and Stein (10) were used for automated amino acid analysis.

The molecular weight of the casein (I, IV, VI) was derived from interference patterns of sedimentation equilibrium experiments in the ultracentrifuge at 4° and a rotor speed of 33,450 rpm. The measurements were made in 0.1 M NaCl–NaOH, pH 10, at a concentration of 0.6–0.7 mg/ml. Other experimental details are described elsewhere (11).

The solubility of the caseins in the presence of calcium ions was observed as follows. Each purified casein was dissolved in enough 0.02 N NaOH to give a solution at pH 6.8, and M $CaCl_2$ was added

[1] Agricultural Research Service, U. S. Department of Agriculture.

TABLE I

SEPARATION AND PURIFICATION OF CASEIN COMPONENTS BY STEPWISE CHROMATOGRAPHY AT 3° ON COLUMNS (2 × 52 cm) OF DEAE–CELLULOSE[a]

First fractionation		Rechromatography of fractions		pH for repre-cipitation of I–VI	Approx. percentage in casein based on protein recovered
Phosphate buffer	Component eluted	Phosphate, pH 8.3	Component		
0.005 M, pH 8.3	VI	0.005 M	VI	5.2	3
0.02 M, pH 8.3	V	0.02 M	V	5.2	8
0.05 M, pH 8.3	1st peak, IV + III	0.04 M	1st peak, IV	4.9	20
—	—	0.04 M	2nd peak, III	4.9	6
0.05 M, pH 8.3	2nd peak, II	—	—		
0.10 M, pH 8.3	II + I	0.06 M	II	4.9	35
—	—	0.075 M	I	4.5	3
0.10 M, 0.3 M NaCl, pH 6.5	II + I	—	—		
0.10 M, 4 M urea, pH 6.5	κ-fraction	—	—		25

[a] Second peak, II, obtained in the first fractionation, was pure and did not require rechromatography. Fraction II + I, obtained with 0.3 M NaCl, pH 6.5, contained some κ-fraction; it was not purified further. The first fractionation of about 2 g casein (dissolved in 40 ml buffer) was made using fibrous DEAE–cellulose and a flow rate of 72 ml/hr. Rechromatography of fractions (about 100–300 mg in 8–12 ml) was with the microgranular material at a flow rate of 24 ml/hr; in each case the starting buffer was at a molarity somewhat lower than that shown except for VI. After rechromatography of the fractions from one sample of casein, indications of proteolysis (the appearance of several new bands in the electrophoretic patterns) were observed. Accordingly, these fractions were chromatographed once again after they were heated at pH 8.3 to 100° for 5 min. New bands were not evident after the heat treatment, and the composition and mobility of the purified components were unaffected.

to a concentration of 0.2 M Ca^{2+}. The final solutions contained 0.8% protein at pH 6.4–6.5. Whether precipitation occurred was noted at 3 and 25°.

Stabilization (solubilization) by the κ-fraction of the calcium-sensitive caseins (those precipitated in the presence of Ca^{2+}) was measured by the method used by Malpress and Seid-Akhavan (3) with minor modifications. In these experiments the concentration of Ca^{2+} was 0.01 M. Stabilized caseins were tested at pH 6.4 for coagulability by rennin.

RESULTS AND DISCUSSION

Figure 1 compares the disc gel electrophoretic pattern at pH 9.6 of casein isolated from human mature milk (gel a) with that from cow milk (gel b). The pattern for human casein has four minor and two major bands. The casein with the fastest mobility is identified as I. The mobilities of caseins I–VI fall in a narrow range between those of bovine γ- and β-caseins. A κ-casein component, though present, is not clearly seen in either case. Patterns of caseins from mature milk of seven different individuals show no variation in the relative intensity of the

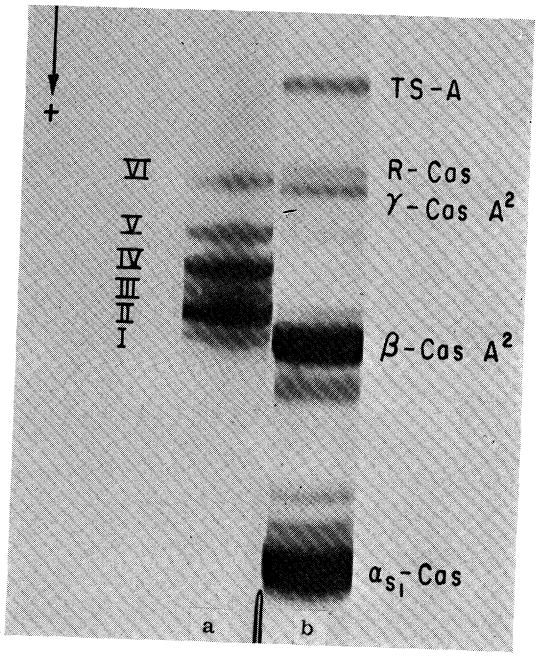

FIG. 1. Disc-gel electrophoresis (pH 9.6, 4 M urea) of human casein, gel a, and cow casein, gel b.

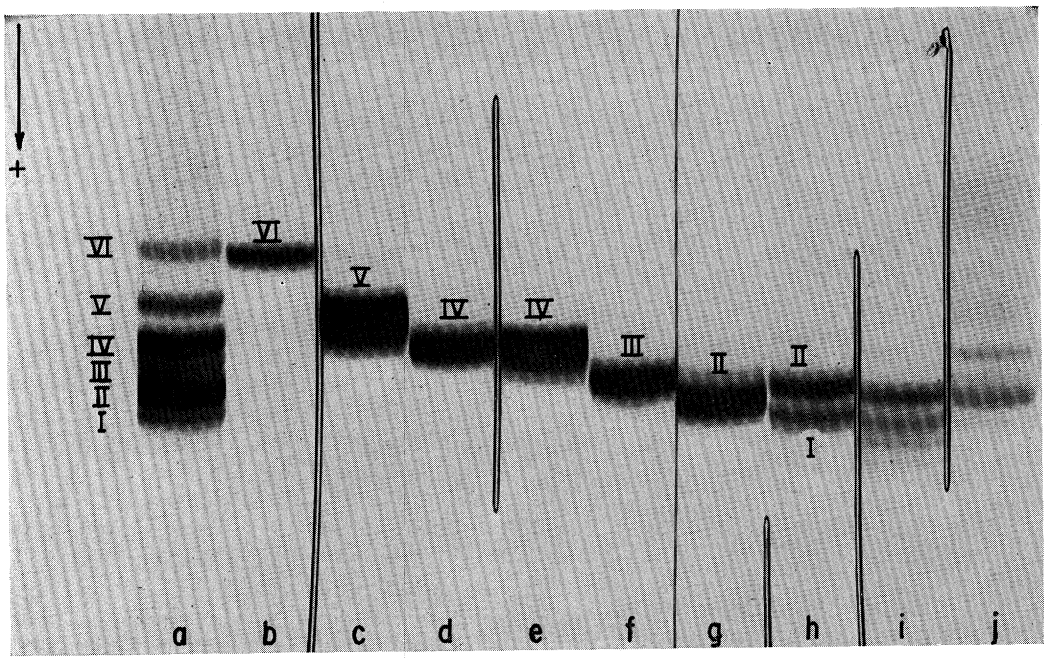

FIG. 2. Disc-gel electrophoresis (pH 9.6, 4 M urea) of human casein, gel a, and the various fractions (gel b–j) obtained by DEAE–cellulose chromatography.

TABLE II

COMPOSITION OF CASEINS

	Composition of human caseins Residues amino acid/molecule								Composition of cow caseins Residues amino acid/molecule		
	Calculated from mean molar ratios based on Arg 3, Ala 7, Phe 5										
	I[a]	II[a]	III[a]	IV[a]	V[a]	VI[a]	Whole number residues	κ-Fr.[b]	β-Cas A[2]	α_{s1}-Cas B	κ-Cas B-1
Lys	11.0	10.8	11.2	11.0	10.9	11.1	11	9.0	11	17	9
His	4.7	4.7	4.7	4.8	4.7	4.9	5	4.7	5	6	3
Amide NH_3	31.6	31.5	32.3	32.9	30.9	30.5	32	4.0	—	—	—
Arg	2.9	2.9	2.9	2.9	2.9	2.9	3	9.1	4	7	5
Asp	11.2	11.2	11.0	11.4	11.3	11.3	11	18.0	9	18	11
Thr	8.7	9.0	8.9	9.0	9.0	9.0	9	9.4	9	6	13
Ser	8.8	8.5	8.7	8.7	8.7	9.0	9	12.8	15	17	12–13
Glu	39.1	38.9	39.0	39.5	39.1	39.4	39	35.3	39	46	27
Pro	38.9	39.0	38.3	40.0	38.7	40.1	39	24.8	34	20	20
Gly	3.2	3.2	3.4	3.2	3.1	3.2	3	3.9	5	11	3
Ala	7.1	7.2	6.9	7.1	7.1	7.1	7	11.6	5	11	14
½ Cys	0	0	0	0	0	0	0	>1.0	0	0	2
Val	19.4	18.9	19.0	19.3	19.2	20.0	19	12.8	18	13	11
Met	3.0	3.2	3.0	2.9	3.0	3.2	3	5.4	6	6	2
Ile	12.8	12.9	12.9	12.9	12.8	13.1	13	10.8	10	13	12
Leu	25.8	25.8	26.4	26.0	26.1	26.3	26	16.2	21	20	8
Tyr	6.9	6.8	6.8	6.9	6.9	6.8	7	9.4	4	12	8
Phe	5.1	5.1	5.2	5.1	5.1	5.1	5	5	9	10	4
Trp	1	1	1	1	1	1	1	—	1	2	1
P	5	4	3	2	1	0		—	5	9	2

[a] Residue numbers listed are averages of, or extrapolated values from, six determinations. Duplicate analyses were made on samples hydrolyzed 24, 72, and 96 hr. The threonine, serine, and amide ammonia numbers were obtained by linear regression analysis.

[b] Analysis of the κ-fraction is that of a single 24-hr hydrolyzate and is based on phenylalanine taken as 5; threonine and serine are uncorrected.

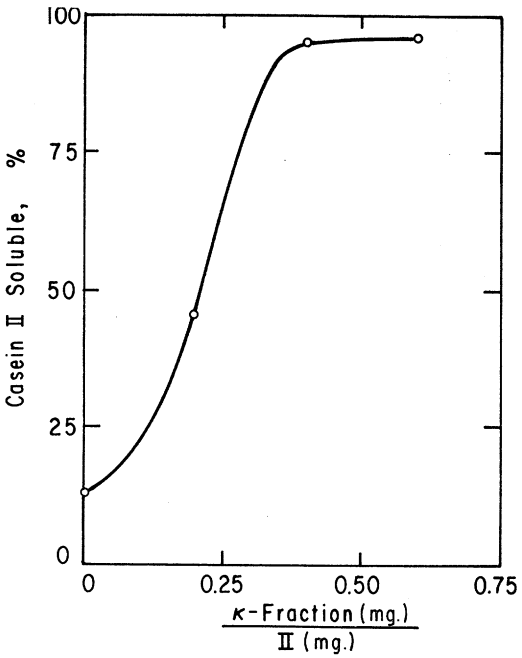

FIG. 3. Stabilization of casein II by κ-fraction.

six bands but a few of the samples do show one or two faint bands of mobility less than casein VI. Malpress and Hytten (2) examined a larger number of individual samples and suggested that the caseins are of three different types. We could not confirm this finding.

Figure 2 shows electrophoretic patterns for the unfractionated human casein (gel a), and for the purified, component caseins (gels b, c, d, e, f, and g). The patterns for the purified caseins I and II, prepared from the 0.1 M phosphate fraction (gel h) are not shown, but they indicate equal purity. Gel i represents that fraction (Table I) eluted from DEAE–cellulose at 0.1 M phosphate, 0.3 M NaCl, pH 6.5, and gel j, the κ-fraction. The gel electrophoretic patterns were compared both before and after reduction of the proteins with 2-mercaptoethanol. In contrast to the effect of reduction on cow casein, in which the diffuse κ-casein zone is changed to several discrete zones after reduction, no new bands were observed for human casein. However, Malpress and Hytten (2) observed a sharpening of bands after reduction of their preparation of human κ-casein.

The amino acid composition and phosphorus content of the purified human caseins are shown in Table II. Results obtained with a 24-hr hydrolyzate of the κ-fraction, based on phenylalanine as 5, as well as analyses of cow β-casein (12), α_{s1}-casein (13) and κ-casein (14), are included for comparison. It was evident from early analyses that caseins I–VI did not differ in amino acid composition. From their averaged tryptophan content of 0.73% (range, 0.67–0.77) a minimum molecular weight of 28,000 was calculated. The numbers (Arg 3, Ala 7, Phe 5) selected for calculation of residue numbers from molar ratios are compatible with a molecule of this size. Adding the residue weights of the whole numbers listed gives a molecular weight of 23,755 for these caseins, a value in excellent agreement with those from sedimentation equilibria, 25,600 for I, 25,000 for IV, and 24,600 for VI. As shown in the table, the distinguishing feature in caseins I–VI is their content of phosphorus. This will be discussed presently. In distribution of amino acids these caseins are more like bovine β-casein than α_{s1}- or κ-caseins.

The preliminary analysis of the unpurified κ-fraction indicates an amino acid composition substantially different from that of caseins I–VI. It may be noted that this fraction contains some half-cystine residues, for cysteic acid was found in hydrolyzates of performic acid-oxidized whole casein and of κ-fraction; none was found in oxidized casein II. The κ-fraction is also probably richer in tryptophan since 0.86% was found in whole casein.

Caseins I through VI contained 0.63, 0.48, 0.41, 0.27, 0.14, and 0.02% P, respectively, or 4.9, 3.7, 3.2, 2.1, 1.1, and 0.15 P residues per molecule (*ca.* 24,000 daltons), normalized in Table II to whole numbers. The stepwise variation, presumably in phosphoserine or phosphothreonine residues, accounts for the differences in electrophoretic mobility illustrated in the figures. The precipitability of these caseins by calcium ions (0.2 M, pH 6.8, 25°) is also apparently dependent on phosphorus content since caseins I, II, and III precipitate heavily under these conditions, IV precipitates slightly, and V and VI are soluble. At 3°, the precipitates dissolve.

Although we were not able at this time to carry further the purification of the κ-fraction, it is obvious that there is in human casein a component similar in properties, and somewhat similar in composition, to bovine κ-casein. Thus, the κ-fraction will stabilize casein II in the presence of 0.01 M calcium ion, as shown in Fig. 3. Furthermore, such stabilized solutions are coagulable by rennin. These results are qualitatively similar to the observations of Malpress and Seid-Akhavan (3) and of Alais and Jollès (15).

Our results do not, of course, rule out the existence of other components of human casein. But we believe that the six major bands seen after gel electrophoresis of human casein represent the same calcium-sensitive protein phosphorylated at different levels. It is of interest that phosvitins prepared from hen eggs and ling roe consist also of mixtures of the same protein phosphorylated, apparently in a stepwise manner, to different levels (16). In amino acid composition caseins I–VI resemble bovine β-casein more closely than α_{s1}-casein, while a kappa component functions as a micelle-stabilizing agent in both systems.

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ADDENDUM

After the completion of this phase of our research and the preparation of the manuscript there have been published two additional papers on human casein by Nagasawa *et al.* In the first (17) the amino acid compositions of colostral, transitional, and mature human milk caseins are compared. In the second (18) the isolation, composition, and properties of a component of mature casein, designated human β-casein B, are described. This component was found to contain 0.41% phosphorus. In all probability our Casein II is the same protein, for amino acid compositions and relative electrophoretic mobilities are similar; however, Casein II contains 0.48% phosphorus.

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